GLP-1 Receptor Agonist Exenatide Increases Capillary Perfusion Independent of Nitric Oxide in Healthy Overweight Men

Mark M. Smits, Marcel H.A. Muskiet, Lennart Tonneijck, Mark H.H. Kramer, Michaela Diamant,† Daniël H. van Raalte,⁎ Erik H. Serné*  

Objective—The insulinotropic gut–derived hormone glucagon-like peptide-1 (GLP-1) increases capillary perfusion via a nitric oxide–dependent mechanism in rodents. This improves skeletal muscle glucose use and cardiac function. In humans, the effect of clinically used GLP-1 receptor agonists (GLP-1RAs) on capillary density is unknown. We aimed to assess the effects of the GLP-1RA exenatide on capillary density as well as the involvement of nitric oxide in humans.  

Approach and Results—We included 10 healthy overweight men (age, 20–27 years; body mass index, 26–31 kg/m²). Measurements were performed during intravenous infusion of placebo (saline 0.9%), exenatide, and a combination of exenatide and the nonselective nitric oxide–synthase inhibitor l-N⁶-monomethyl arginine. Capillary videomicroscopy was performed, and baseline and postocclusive (peak) capillary densities were counted. Compared with placebo, exenatide increased baseline and peak capillary density by 20.1% and 8.3%, respectively (both P=0.016). Concomitant l-N⁶-monomethyl arginine infusion did not alter the effects of exenatide. Vasomotion was assessed using laser Doppler fluxmetry. Exenatide nonsignificantly reduced the neurogenic domain of vasomotion measurements (R=−5.6%; P=0.092), which was strongly and inversely associated with capillary perfusion (R=−0.928; P=0.036). Glucose levels were reduced during exenatide infusion, whereas levels of insulin were unchanged.  

Conclusions—Acute exenatide infusion increases capillary perfusion via nitric oxide–independent pathways in healthy overweight men, suggesting direct actions of this GLP-1RA on microvascular perfusion or interaction with vasoactive factors. (Arterioscler Thromb Vasc Biol. 2015;35:1538-1543. DOI: 10.1161/ATVBAHA.115.305447.)  

Key Words: capillaries ‖ glucagon-like peptide-1 ‖ nitric oxide

G lucagon-like peptide-1 (GLP-1) is a gut-derived hormone, secreted after food intake. GLP-1 potently stimulates glucose-stimulated insulin secretion, while inhibiting glucagon secretion, thereby lowering blood glucose levels.1 However, GLP-1 is rapidly inactivated by the enzyme dipeptidyl peptidase-4, resulting in a short half-life of 2 minutes.1 Degradation-resistant antihyperglycemic drugs that mimic the effects of native GLP-1, so-called GLP-1 receptor agonists (GLP-1RAs), have recently been developed and are now commonly prescribed in type 2 diabetes mellitus treatment. It has been demonstrated that native GLP-1 has various effects on vascular and endothelial function.2 Interestingly, infusion of GLP-1 increases microvascular blood flow in rats and humans.3–5 In animals, these microvascular effects of native GLP-1 increase insulin and glucose delivery to muscle interstitium, and enhance insulin-independent glucose uptake in muscle.3,5,6 In addition, this increase in microvascular perfusion is a probable explanation of the improved myocardial function observed after GLP-1 infusion.3,7 Vascular actions of GLP-1 seem to be effectuated through nitric oxide (NO)–dependent mechanisms, as was shown in endothelial cells8,9 and during coinfusion with a NO-synthase inhibitor in male rats.8 Although microvascular effects of native GLP-1 are relevant from a physiological perspective, hitherto no studies with clinically used GLP-1RA on human microcirculation have been performed. Exenatide, the first available and commonly used GLP-1RA, mimics many, yet not all, effects of the GLP-1 peptide.10,11 Thus, although GLP-1 improves microvascular perfusion, it is presently unclear whether this holds true for exenatide. Therefore, in the current clinical mechanistic study, we aimed to assess the effects of the GLP-1RA exenatide on microvascular perfusion in healthy overweight volunteers. In addition, given the NO-dependency of GLP-1–induced microvascular effects in animals, we used the nonspecific NO-synthase inhibitor l-N⁶-monomethyl-arginine (l-NMMA) to examine the potential role of NO as mediator of the vascular and metabolic actions of exenatide in humans.
Nonstandard Abbreviations and Acronyms

<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>GLP-1</td>
<td>glucagon-like peptide-1</td>
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<td>GLP-1RA</td>
<td>glucagon-like peptide 1 receptor agonist</td>
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<td>HR</td>
<td>heart rate</td>
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<tr>
<td>L-NMMA</td>
<td>L-arginine monomethyl ester</td>
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<td>NO</td>
<td>nitric oxide</td>
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<td>SNS</td>
<td>sympathetic nervous system</td>
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Materials and Methods

In this mechanistic cross-over study, healthy overweight participants were subjected to continuous intravenous infusion of placebo (isotonic saline), GLP-1RA exenatide, L-NMMA or a combination of L-NMMA and exenatide. During these conditions, microvascular perfusion was assessed using intravital videomicroscopy and laser Doppler fluxmetry. A complete description of Materials and Methods are available in the Online-only Data Supplement.

Patient Characteristics

Ten healthy overweight men were included in this analysis. Full characteristics of the subjects can be found in the Table.

Capillary Videomicroscopy

Compared with placebo, exenatide increased baseline capillary density with 8.0 (5.3–10.5) cap/mm² (P=0.016) and peak postarterial occlusion capillary density with 6.5 (4.8–7.8) cap/mm² (P=0.016). This corresponds with an increase of 20.1% and 8.3%, respectively. When exenatide was confounded with L-NMMA, no significant differences compared with exenatide infused alone were observed (Figure 1).

Tests were performed during 2 study days. Day-to-day variation for baseline capillary density and postocclusive peak density were 4.7% and 1.7%, respectively. We conducted sensitivity analyses to assess potential interference of day-to-day variation by including unstimulated measurements (before any intervention) in the statistical model. These sensitivity analyses showed no different results compared with the original analyses.

Results

Table. Subject Characteristics in Median (Interquartile Range)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Median (IQR)</th>
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<tbody>
<tr>
<td>Age, y</td>
<td>22 (22–23.3)</td>
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<tr>
<td>BMI, kg/m²</td>
<td>29.3 (28.1–30.9)</td>
</tr>
<tr>
<td>Waist circumference, cm</td>
<td>103 (97.5–105.2)</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>112.8 (110.6–127.6)</td>
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<tr>
<td>Diastolic blood pressure, mmHg</td>
<td>65 (62.8–69.1)</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>59 (54.4–66.1)</td>
</tr>
<tr>
<td>Fasting plasma glucose, mmol/L</td>
<td>5.0 (4.7–5.1)</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>0.7 (0.6–1.8)</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>1.3 (0.6–1.9)</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>4.5 (3.7–5.0)</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.2 (1.1–1.3)</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L</td>
<td>2.7 (2.0–3.2)</td>
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BMI indicates body mass index; HDL, high-density lipoproteins; HOMA-IR, homeostatic model assessment–insulin resistance; and LDL, low-density lipoproteins.

Laser Doppler Fluxmetry

During placebo infusion, microvascular perfusion was predominantly determined by power in the lower spectra (0.01–0.15 Hz; Figure 2). Data on exenatide was only available for 6 subjects, showing no effect on skin blood flow or any of the specific vasomotion frequency bands. However, a nonsignificant trend toward an exenatide-induced decrease in the neurogenic domain was observed (R=−5.6%; P=0.092).

Blood Pressure

During placebo infusion, systolic blood pressure was 113.8 (106.0–122.0) mmHg, diastolic blood pressure was 67.8 (60.1–72.3) mmHg, and heart rate (HR) was 59.0 (52.5–64.6) bpm. Exenatide showed no effect on systolic blood pressure or diastolic blood pressure, but significantly increased HR with 5.0 (0.3–12.0) bpm (P=0.038). The combination of exenatide/L-NMMA led to higher systolic blood pressure (P=0.024) and HR (P=0.01) compared with exenatide alone.

Metabolic Parameters

Exenatide lowered glucose from 4.3 (4.3–4.4) mmol/L during placebo to 3.8 (3.7–3.9) mmol/L (P=0.024; Figure 3). No significant difference was seen between exenatide and the combination of exenatide/L-NMMA. Insulin levels were not
altered by exenatide (29.8 [22.9–45.0] pmol/L) versus placebo (27.7 [21.4–51.6] pmol/L).

Correlations
Because exenatide-induced changes in capillary density can be explained by changes in HR, glucose, or fluxmetry, we performed univariable analyses to identify potential correlations. Exenatide-induced changes in the neurogenic domain of vasomotion were inversely associated with exenatide-induced change in peak postocclusive capillary density ($R=−0.928; P=0.032$; Figure 4). No correlations between changes in HR, glucose, or insulin and changes in microcirculatory measurements were found.

Single $\text{-NMMA}$ Infusion
Although not part of the original research question, we explored the effects of $\text{-NMMA}$ infusion alone. During capillary videomicroscopy, $\text{-NMMA}$ had no effect on baseline capillary density, whereas a trend toward a decrease in peak postocclusive density was seen (−5.5 [−9.8 to −1.8] cap/mm²; $P=0.056$). Laser Doppler signal was not affected by $\text{-NMMA}$. $\text{-NMMA}$ did not alter systolic blood pressure, but increased diastolic blood pressure by 7.5 (3.9–10.8) mm Hg ($P=0.016$). HR decreased nonsignificantly with $\text{-NMMA}$ (−7.0 [−8.0 to −1.6] bpm; $P=0.118$). $\text{-NMMA}$ infusion did not change plasma glucose or insulin levels.

Discussion
In this mechanistic study, we demonstrate that the GLP-1RA exenatide increases capillary density, that is, microvascular perfusion, in healthy overweight men. These findings are consistent with several recent studies using native GLP-1 infusion in animals and humans, in which an increase in microvascular perfusion was demonstrated. We are the first to demonstrate that this vasoactive effect is also present with the use of a GLP-1RA in humans. Moreover, in contrast to previous findings using native GLP-1, we demonstrate that the microvascular effects of exenatide are independent of NO and insulin concentrations.

Capillary perfusion is regulated by precapillary arteriolar tone, which is modulated by several systemic and local factors. We demonstrate that exenatide increases perfusion, which can either be a direct or an indirect effect. A direct effect is conceivable, and can be explained by the observation that the GLP-1 receptor is present on smooth muscle cells of human arteries. Alternatively, exenatide could interact with factors known to alter precapillary arteriolar tone, an indirect effect. NO and atrial natriuretic peptide lead to vasodilation, which increases microvascular perfusion. In contrast, other factors (eg, endothelin-1, the sympathetic nervous system (SNS), and renin–angiotensin–aldosterone system) are known to cause vasoconstriction, thereby reducing microvascular perfusion. The effects of exenatide persisted during blockage of the synthesis of NO, suggesting a NO-independent pathway. In a mouse model, GLP-1 receptor activation increased atrial natriuretic peptide secretion. In patients with type 2 diabetes mellitus, the GLP-1RA liraglutide also increased atrial natriuretic peptide levels after 12 weeks of treatment, although results in humans are inconsistent. Liraglutide was furthermore shown to inhibit the effects of endothelin-1 in cultured endothelial cells. Finally, exenatide reduced the effects

Figure 2. Fluxmetry during placebo, exenatide, and $\text{-NMMA}$. Fluxmetry as assessed using laser Doppler. Exenatide had no significant effect on total power or any of the domains. (*a trend toward an exenatide-induced decrease in neurogenic domain was observed, $P=0.096$).

Figure 3. Glucose and insulin levels during infusion with placebo, exenatide, and $\text{-NMMA}$. Levels of glucose and insulin. Exenatide infusion significantly decreased glucose levels. This effect was not altered by coinfusion of $\text{-NMMA}$. No effect of exenatide was seen on insulin levels. $P<0.05$ compared with placebo.
of angiotensin-II in a hypertensive mouse model, suggestive of anti-rexin–angiotensin–aldosteron system effects, yet clinical studies using GLP-1(RA) are inconclusive. Thus, GLP-1(RA) seem to have effects on many factors known to influence precapillary arteriolar tone, and as such, could alter capillary perfusion.

In this study, we have shown that exenatide-induced actions on skin microvascular perfusion are independent of NO-availability. This observation is in line with findings from isolated rat femoral arteries, in which GLP-1 dose-dependently induced vasodilation in the presence of a NO-synthase blocker and after removal of endothelium. The latter suggests that the vasodilator effects of GLP-1 are independent of any endothelial-derived substance. However, other preclinical studies contrast our findings in humans. As such, infusion of the native GLP-1 was shown to increase muscle microvascular perfusion in several animal studies, whereas effects were abolished when NO-synthase was inhibited. The observed discrepancy in NO-dependency remains to be elucidated. Feasibly, NO-dependence may differ between different types of vasculature. However, both skin and muscle microcirculation are affected by blockage of NO synthase. Another explanation could be that exenatide, which is based on a peptide derived from the venom of the gila monster (Heloderma suspectum), only shares 53% of amino acid homology with GLP-1, potentially leading to activation of NO-independent pathways.

Microvascular blood flow is known to undergo rhythymical variations or vasomotion. Vasomotion activity in the microvascular bed can be explored by analysis of the component frequencies of the laser Doppler signal. Distinct periodic oscillations in the laser Doppler signal have been attributed to, consecutively, the heartbeat, respiration, myogenic activity in the vessel wall, neurogenic activity, and endothelial activity. Especially, the neurogenic domain of vasomotion is affected by insulin and closely relates to capillary perfusion in humans. Exenatide also seemed to influence the neurogenic domain, although results did not reach statistical significance. Interestingly, changes in the neurogenic domain were strongly associated with an increase in capillary density. Because a decrease in the neurogenic domain is associated with a decrease in SNS activity, it could be speculated that exenatide decreases dermal vascular SNS activity, leading to a reduced precapillary arteriolar tone and increased microvascular perfusion. Because the SNS directly affects smooth muscle cells in the arterial wall, endothelial cells may not be required for this vasoactive effect. Also, as no effects on the endothelial domain were seen, exenatide-induced effects may be independent of the vascular endothelium.

Exenatide infusion had no effect on insulin levels in this study. It is known that the actions of exenatide on insulin secretion are glucose-dependent, occurring only during hyperglycemia. Because our subjects were normoglycemic, this could reduce the effect on insulin secretion. In addition, single measurement of insulin levels was performed 2 hours after the start of exenatide infusion. In previous studies, no effect of exenatide on fasting insulin levels were seen ≤105 minutes of infusion. Because the effects of exenatide on microvascular perfusion occurred during a period where insulin levels were unchanged, we could argue that observed effects are insulin-independent. This hypothesis is in line with several previous studies. In a rat study, GLP-1 increased muscle blood flow during a euglycemic–hyperinsulinemic clamp, whereas the peptide increased muscle microvascular perfusion during infusion with ocreotide in healthy men. Interestingly, the effect of exenatide on capillary density (≤20% increase) seems to be more pronounced than the effect obtained with systemic hyperinsulimenia (≤9% increase).

The exenatide-induced increase in microvascular perfusion can be clinically relevant because capillaries are located throughout all organ tissues. For instance, increased microvascular perfusion in muscle could lead to increased glucose uptake, a beneficial phenomenon which was shown in rodents and dogs. In our study, we observed insulin-independent reductions in glucose levels after exenatide infusion, which could be explained by increased muscle glucose disposal rate. However, in this study, we did not assess this by using label-tracer techniques. Clinically, improved microvascular perfusion may favorably affect myocardial function, as was recently demonstrated for exenatide in 8 insulin-naive type 2 diabetes mellitus patients. However, in this study, a positron emission tomography assessment of myocardial perfusion was used, which is not able to differentiate between macro- and micro-circulation. Future studies should assess whether beneficial
effects of GLP-1RA are effectuated through increased microvascular perfusion.

This study has some limitations. First, the study had a nonrandomized and nonblinded design. However, the investigator was blinded for the status of the recordings, thereby eliminating the potential of bias when counting the number of capillaries. Second, the studied population is not the population treated with exenatide in clinical practice, indicating the need for further studies in patients with type 2 diabetes mellitus. However, the long-acting GLP-1RA lixisenatide is now registered as weight management agent in obese nondiabetic individuals. Thus, examining the microvascular effects in this population has clinical relevance. Finally, data of only 6 participants were available for the vasomotion analysis of exenatide, introducing the possibility of type II errors. For our primary analysis, exenatide-induced changes in capillary perfusion as assessed using capillary videomicroscopy, we had statistical power of 1.000. Contrary to previous studies, we investigated the effects of GLP-1RA on skin microcirculation. Given that muscle cells are involved in glucose uptake, the actions of exenatide on muscle microcirculation might metabolically be more relevant. However, in previous studies, we demonstrated that the cutaneous microcirculation is a representative vascular bed to examine insulin’s actions on the microcirculation. Moreover, assessment of the effects of exenatide on skin microvasculature has 2 benefits. First, in contrast to measurements of muscle microcirculation, direct capillary videomicroscopy allowed us to assess the effects of exenatide not only on microvascular perfusion but also on functional capillary capacity. Second, the skin enabled us to study vasomotion, the spontaneous rhythmic change of arteriolar diameter. Using vasomotion, it is possible to assess the amount of microvascular perfusion, as well as endothelial, neurogenic, and myogenic influences.

In conclusion, we demonstrated that the GLP-1RA exenatide increases capillary perfusion in skin, independent of NO. In addition, our data suggest that the effects are independent of insulin, and could potentially be explained by a decrease in SNS activity.

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Disclosures
Before her passing on April 9, 2014, M. Diamant was a consultant for Abbott, Astra Zeneca, Boehringer–Ingelheim, Bristol-Myers Squibb, Eli Lilly, GI Dynamics, Merck Sharp & Dohme, Novo Nordisk, Poxel Pharma, and Sanofi. She was a speaker for Astra Zeneca, Bristol-Myers Squibb, Eli Lilly, Novo Nordisk, and Sanofi and through MD, the VU University Medical Centre received research grants from Abbott, Astra Zeneca, Boehringer–Ingelheim, Bristol-Myers Squibb, Eli Lilly, Medtronic, Merck Sharp & Dohme, Novo Nordisk, and Sanofi. MD received no personal payments in connection to the abovementioned activities: all funds are directly transferred to the Diabetes Center’s nonprofit Research Foundation. The other authors report no conflicts.

References
We have demonstrated that the glucagon-like peptide-1 receptor agonist exenatide, a commonly prescribed glucose-lowering agent, increases microvascular perfusion independent of nitric oxide. In animal models, an increase in microvascular perfusion is known to improve muscle glucose uptake and myocardial function. Future studies should assess whether the microvascular effects of glucagon-like peptide-1 receptor agonists are beneficial for patients with type 2 diabetes mellitus.
GLP-1 Receptor Agonist Exenatide Increases Capillary Perfusion Independent of Nitric Oxide in Healthy Overweight Men

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Materials and Methods

In this mechanistic cross-over study, healthy overweight participants were subjected to continuous intravenous (IV) infusion of placebo (isotonic saline), GLP-1RA exenatide, L-NMMA or a combination of L-NMMA and exenatide. During these conditions, microvascular perfusion was assessed using capillary videomicroscopy and laser Doppler fluxmetry.

Participants
Ten healthy overweight (body mass index (BMI) >25 kg/m²) male subjects, aged 18-30 years, were recruited by advertisements. Participants were not allowed to use any type of medication. During a screening visit, blood and urine samples were taken to ensure a healthy condition. In addition, a 2-hour 75 mg oral glucose tolerance test was performed to confirm normal glucose metabolism, defined as fasting glucose < 5.6 mmol/l, and < 7.8 mmol/l 2 hours after the oral glucose load. Subjects with a history of working nightshifts, smoking or excessive alcohol use (>3 units/day) were excluded. The study was approved by the local ethics review board, and was conducted according to the Declaration of Helsinki and guideline for good clinical practice. All subjects provided written informed consent before participation.

Study Design
This was a non-blinded, non-randomized, cross-over study performed over 2 separate testing-days, which were planned in no particular order. During one visit, the acute effects of IV placebo and subsequently exenatide were measured. During the other visit, the acute effects of IV L-NMMA and a combination of L-NMMA and exenatide were assessed.

Two days prior to both study visits, subjects were instructed to adhere to a normal-salt (9-12 grams per day) and normal-protein (1.5-2 mg/kg per day) diet. All participants abstained from heavy exercise (24 hours), alcohol (24 hours) and caffeine (12 hours) prior to the study visit. After an overnight fast, subjects arrived at the clinical research unit at 07.30 AM. IV catheters were placed in both forearms, and subjects were instructed to assume a semi-recumbent position throughout the testing-day. After 30 minutes of acclimatization, the study tests were started. All measurements were performed in a temperature-controlled room (23.0 ± 1.0 °C). Skin temperature was registered continuously and was above 28 °C at the start of all microvascular measurements.

Prior to the start of any intervention, baseline blood pressure measurements were performed using an automated oscillometric device (Dinamap®, GE Healthcare, Little Chalfont, United Kingdom; UK). Microvascular perfusion was assessed by capillary videomicroscopy and laser Doppler fluxmetry in the skin (see below). Then, either placebo or L-NMMA was started, and after 60 minutes blood was drawn for metabolic parameters and cardiovascular tests (blood pressure, capillary videomicroscopy and laser Doppler fluxmetry) were repeated. After the cardiovascular test, another blood sample was drawn. On both study days, exenatide was subsequently infused, with cardiovascular measurements repeated after 120 minutes, approximately 60 minutes after reaching an assumed equilibrium. During the testing day, subjects received a constant oral water load of 200 mL/hour. An overview of the testing day is visualized in the online supplemental figure I.

Study Drugs
The GLP-1RA exenatide (AstraZeneca, London, UK) was administered intravenously to assess acute effects during a steady state concentration. As validated previously by our group and others, a loading dose of 50 ng/min for 30 minutes, followed by a continuous infusion of 25 ng/min, yields plasma levels within the therapeutic range and harbors a good
tolerability profile. L-NMMA acetate (Bachem GmbH, Weil am Rhein, Germany) was administered intravenously to assess the NO-dependency of the effects of exenatide. After a loading dose of 5 mg/kg for 5 minutes, a continuous infusion of 50 µg/kg/min was given. This dose is known to have vasopressor activity.4

**Capillary Videomicroscopy**

Nailfold capillary videomicroscopy was performed using the VCS Video Capillaroscopy System (KK Technology, Honiton, UK). This system includes a high-quality monochrome CCD camera, resolution 752 x 582 pixels, which uses cold light epi-illumination for high-contrast images. The microscope was coupled to a laptop running CapiScope software version 3.90 (KK Technology, Honiton, UK) for image recording and analysis. The nailfold of the third digit of the non-dominant hand was placed under the microscope at the subjects’ heart level, who remained in a semi-recumbent position. Two separate visual fields of 1 mm² were recorded before and after 4 minutes of arterial occlusion (established by inflating a cuff placed around the base of the finger to 300 mmHg). The same two fields were identified for every measurement. Baseline recordings were analyzed for capillary density, which is the number of capillaries per mm² of nail fold skin that are continuously perfused for 15 seconds.5 The maximum number of capillaries counted directly after release of arterial occlusion defined peak capillary density. While baseline capillary density represents functional capillary perfusion in the resting state, peak capillary density is a measure of capillary reserve capacity.6 The investigator counting the capillaries (MMS) was blinded to the status of the recordings. Second time blinded counting established an intra-observer coefficient of variation of 3.9%.

**Laser Doppler fluxmetry**

A laser Doppler fluxmetry system (Periflux 4000; Perimed, Stockholm, Sweden) was used to measure skin blood flow. Using this technique, perfusion of the microcirculation is expressed in arbitrary perfusion units (PU). Measurements were performed with one thermostatic laser Doppler probe (PF 481; Perimed, Stockholm, Sweden) placed at the dorsal side of the middle phalanx of the middle finger of the dominant hand. Additionally, wavelet analysis of the laser Doppler flux signals (signal = velocity X number of particles) over 30 min was conducted to assess the frequency spectrum between 0.01 and 1.6 Hz. Using Matlab (Version 7.8.0.347; The Mathworks, Inc., Natick, MA, USA), the laser Doppler flux recording spectrum was divided into five frequency intervals as described by Stefanovska et al: 0.01–0.02 Hz (endothelial activity); 0.02–0.06 Hz (neurogenic activity); 0.06–0.15 Hz (smooth muscle response in the vessel wall); 0.15–0.4 Hz (respiratory function); and 0.4–1.6 Hz (heart beat frequency). Since laser Doppler flux signal strength varies between subjects and between measurements, normalized amplitudes were calculated for each of the five frequency bands by dividing the average amplitude within a band by the average amplitude of the entire spectrum. Thus, using laser Doppler fluxmetry, both skin perfusion and vasomotion can be measured.

**Laboratory measurements**

Blood was drawn from the IV catheter using syringes, and immediately transferred to designated BD Vacutainer® tubes (Franklin Lakes, NJ, USA). Throughout the testing day, venous plasma glucose was measured using a YSI 2300 STAT Glucose analyzer (YSI Life Sciences, Yellow Springs, Ohio, USA). Fasting plasma glucose and 2-hour post-OGTT glucose were measured from heparin plasma using the Gluco Quant-hexokinase method on a Modular P (Roche Diagnostics, Basel, Switzerland) within an hour of drawing blood. Triglycerides and total cholesterol were determined using an enzymatic colorimetric method, and high-density lipoprotein (HDL)-cholesterol was assessed using the 3rd generation HDL-C plus method. Low-density lipoprotein (LDL) cholesterol was calculated by Friedewald
Insulin was determined from heparin plasma using an immunometric assay (Advia Centaur XP Immunoassay System, Siemens Healthcare, Erlangen, Germany). During the testing day, blood samples for glucose and insulin were drawn prior to and after the cardiovascular measurements. A mean of the two values was used for statistical analyses.

Statistics
A sample size calculation was performed prior the start of this study. Based on previous clinical data, we expected an increase of at least 30% in microvascular perfusion as assessed using capillary videomicroscopy (standard deviation 30%). Considering an $\alpha$ of 5% and power of 80%, we calculated that 8 subjects would ensure sufficient power. Keeping in mind the potential of drop-outs and failed experiments, we decided to include 10 subjects.

All data are presented as median [interquartile range]. Statistical analyses were performed using non-parametric tests, given the number of subjects and non-Gaussian distribution of the variables, even after log transformation. Wilcoxon signed rank test was used for paired analyses, to identify single effects of exenatide versus placebo, and the combination of exenatide/L-NMMA versus exenatide. Correlation analyses were performed using Spearman’s rank correlation coefficient.

Correction for multiple testing was performed after all tests using the Bonferroni-method. Given that the primary interests were the comparisons of the effects of (1) exenatide vs placebo, and (2) exenatide vs the combination of L-NMMA/exenatide, all statistical results were corrected for multiple testing by multiplying the $p$-value by 2. For the correlation analyses, the $p$-value was multiplied by 4, which equals the amount of performed statistical correlation tests. The analyses with L-NMMA only were considered exploratory, and therefore additional correction for multiple testing was not performed.

Sensitivity analyses were performed to assess day-to-day variation by correcting for unstimulated measurements. A relative variable was calculated by subtracting the unstimulated measurement by the stimulated measurements (placebo, exenatide, L-NMMA and L-LNMMA/exenatide). These relative measurements were then used instead of the original variables for the sensitivity analyses.

A $p$-value <0.05 was considered statistically significant. All tests were performed using SPSS version 20.0 for Windows (IBM SPSS Inc., Chicago, IL, USA).

References


Online Supplementary Figure I
Study Layout

Day 1
- Placebo
- Exenatide
- L-NMMA
- Exenatide / L-NMMA

Day 2
- Test block

Blood drawing
Blood pressure
Laser Doppler Fluxmetry
Capillary Videomicroscopy
Blood drawing

30 min
60 min
30 min
60 min
30 min

30 min
60 min
30 min
60 min
30 min

60 min 'equilibration'
30 min

30 min
60 min
30 min
60 min
30 min

30 min

Blood drawing
Blood pressure
Laser Doppler Fluxmetry
Capillary Videomicroscopy
Blood drawing